

U.S. Army Center for Health Promotion and Preventive Medicine

Wildlife Toxicity Assessment for 1,3-Dinitrobenzene (m-DNB)

DECEMBER 2001

Prepared by
Health Effects Research Program
Environmental Health Risk Assessment Program

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Department of the Army
U.S. Army Center for Health Promotion and Preventive Medicine

Wildlife Toxicity Assessment for m-DNB

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1. INTRODUCTION

1,3-Dinitrobenzene (m-DNB) is one of several compounds that have been released to the environment during the manufacture of explosives and in load, assembly and pack (LAP) activities at U.S. Army ammunition plants (AAPs) and other military installations. The compound has a close structural relationship with the important military explosive trinitrotoluene (TNT), of which m-DNB is a manufacturing by-product and an environmental degradation product. The importance of m-DNB as an environmental contaminant is related to its widespread distribution at and around military sites and to its potential toxicity to wildlife and other ecological receptors. This Wildlife Toxicity Assessment summarizes current knowledge of the likely harmful impacts of m-DNB on wildlife, with emphasis on identifying levels at which wildlife species may be adversely effected. Evaluating the toxicity of the compound is intended to contribute to the establishment of toxicity reference values (TRVs) that could serve as protective exposure standards for wildlife ranging in the vicinity of m-DNB contaminated sites. The protocol for the performance of this assessment is documented in the U.S. Army Center for Health Promotion and Preventive Medicine Technical Guide 254, the *Standard Practice for Wildlife Toxicity Reference Values* (USACHPPM 2000).

2. TOXICITY PROFILE

2.1 Literature Review

Relevant biomedical, toxicological and ecological databases were electronically searched May 17, 2000, using Dialog to identify primary reports of studies and reviews on the toxicology of m-DNB. Separate searches were carried out linking the compound to either laboratory mammals, birds, reptiles and amphibians (combined) and wild mammals. In general, a two-tiered approach was used in which all citations were first evaluated as titles and “key words in context.” All available abstracts of those articles that were selected in the first tier as possibly relevant to TRV development were then evaluated for relevancy and retention for evaluation in the second tier. For m-DNB, 30 articles were marked for

retrieval from 62 initial hits. Details of the search strategy and the results of the search are documented in Appendix A.

In addition to literature searches using Dialog, a number of U.S. Army reports were identified in the Defense Technical Information Center (DTIC). Secondary references and sources of information on 1,3-dinitrobenzene (m-DNB) included an Agency for Toxic Substances and Disease Registry (ATSDR) *Toxicological Profile for 1,3-Dinitrobenzene 1,3,5-Trinitrobenzene* (ATSDR, 1995), the National Library of Medicine's Hazardous Substances Databank (HSDB, 2000), the U.S. Environmental Protection Agency's (U.S. EPA) Integrated Risk Information System (IRIS) (U.S. EPA, 2000) and Health Effects Assessment Summary Tables (HEAST) (U.S. EPA, 1997).

2.2 Environmental Fate and Transport

m-DNB has been used as a component in the chemical synthesis of (1) m-nitroaniline, an intermediate in the production of aniline dyes and (2) m-phenylenediamine, a compound used in the synthesis of aramid fibers and spandex (ATSDR, 1995). As with its structural analog 1,3,5-trinitrobenzene (1,3,5-TNB), m-DNB is a manufacturing by-product of the explosive TNT, with the potential for release to the environment in discharged wastewater. Additionally, any 2,4-dinitrotoluene (2,4-DNT) present in the waste stream may be degraded to m-DNB by photolysis under certain pH conditions and organic matter content (as reported in Talmage et al., 1999). Incidental release to the environment of m-DNB might be as a result of any or all of the manufacturing processes referred to above. Soil concentrations of up to 45.2 mg m-DNB/kg soil have been reported at contaminated sites such as AAPs (Talmage et al., 1999).

A list of key physico-chemical properties of m-DNB that pertain to the environmental fate and transport of the compound is provided in Table 1.

m-DNB has an estimated vapor pressure of 5.13×10^{-6} mm Hg at 25°C (ATSDR, 1995), a low value implying that partitioning to air is unlikely. Although readily soluble in a variety of organic solvents at ambient temperature, m-DNB is sparingly soluble in water (370–500 mg/L at 20–25°C). However, despite its limited solubility, the compound has been identified in both surface water and groundwater. Furthermore, significant concentrations in sediments have been identified in streams contaminated with m-DNB. Talmage et al. (1999) present m-DNB soil concentration data for a selection of AAPs, depots and arsenals.

As noted in ATSDR (1995), there are no experimental data on the photolysis of m-DNB in aqueous solution but, on theoretical grounds, the process may be expected to occur, given the potential for the compound to absorb light at wavelengths > 290 nm. Log K_{oc} values have been estimated within the range 1.39 – 2.3, which indicate a moderate degree of adsorption of m-DNB to suspended sediments, and high to moderate mobility in soil.

Table 1. Summary of Physical-Chemical Properties of 1,3-Dinitrobenzene

Molecular weight	168.11
Color	yellow-white
Physical state	crystals/rhombohedral plates
Melting point	89–90 °C
Boiling point	300–303 °C
Odor	no data
Solubility	370–500 mg/L in water at 20–25 °C; soluble in chloroform, acetone and ether
Partition coefficients:	
Log K _{ow}	1.49 to 1.62
Log K _{oc}	1.39 to 2.3
Vapor pressure at 25 °C	5.13×10^{-6} mm Hg
Henry's Law constant at 25 °C	2.33×10^{-6} atm.m ³ /mole
Conversion factors	1 ppm = 6.88 mg/m ³ 1 mg/m ³ = 0.145 ppm

Sources: ATSDR, 1995; Talmage et al., 1999; HSDB, 2000; Wentsel et al., 1979

m-DNB is subject to microbial degradation by a variety of microbes that use the compound as a carbon as well as a nitrogen source. This capacity for microbial degradation differs from that of 1,3,5-TNB in which the aromatic six-carbon ring structure is conserved. Various mixed cultures of microorganisms obtained from rivers or sewage sludge effluents have been shown to break down m-DNB. Aerobic degradation of m-DNB to carbon dioxide has been demonstrated with the microbial strain *Candida pucherrima* (Dey and Godbole, 1986). ATSDR (1995) documents a wide range of microbial genera that can break down m-DNB.

2.3 Summary of Mammalian Toxicity

2.3.1 Mammalian Toxicity - Oral

2.3.1.1 Mammalian Oral Toxicity - Acute

The review by Wentsel et al. (1979) contains a summary of earlier studies on the acute oral toxicity of m-DNB in experimental animals. For example, Kiese (1949) derived an oral LD₅₀ of approximately 10 mg m-DNB/kg in dogs. Cody et al. (1981) reported an average (male/female) oral LD₅₀ for m-DNB of 83 mg/kg. In this study, six male and female Carworth Farms rats were administered one of six doses of m-DNB ranging from 36 to 180 mg/kg. The compound was administered as a 1% solution in corn oil via intubation. In another study, FitzGerald et al. (1991) developed average oral LD₅₀s for m-DNB of 59.5 mg/kg in Fischer 344 (F344) rats and 80.4 mg/kg in Swiss mice. Five rats and mice of both sexes were

dosed with one of three levels of m-DNB suspended in corn oil. In rats the doses were 62, 68 and 74 mg/kg while in mice the doses were 50, 70 and 90 mg/kg.

Some studies on acute toxicity of m-DNB have focused on endpoints other than lethality. For example, Watanabe et al. (1976) demonstrated the formation of methemoglobin in blood samples obtained five hours after intraperitoneal injections of male Wistar rats with 100 μ moles/kg m-DNB in 2 ml/kg propylene glycol (PEG). The primary focus of this study was the methemoglobin-forming potential of 1,3-diamino-2,4,6-trinitrobenzene (DATNB), the parallel use of other nitrogen-substituted aromatic compounds, such as m-DNB, serving as reference compounds. Of the compounds employed in this survey, m-DNB was considered one of the most potent inducers of methemoglobin. Myers et al. (1999) administered a single oral dose of 50 mg/kg m-DNB by gavage in corn oil to four male shrews (*Cryptotis parva*) and demonstrated the formation of hemoglobin adducts in blood samples obtained 24 hours after dosing. *In vitro* incubation of blood with m-DNB also resulted in adduct formation, suggesting a role for cysteine residues in hemoglobin-m-DNB binding. This finding was consistent with an earlier *in vitro* demonstration that m-DNB and other DNB isomers can bind irreversibly to red blood cell macromolecules (Cossum and Rickert, 1987).

A number of single, oral dose studies of m-DNB have provided information regarding the biochemistry and toxicokinetics of neurotoxicological and testicular impairment induced by the compound. For example, Philbert et al. (1987a) used germ-free (GF) and conventional male F344 rats to demonstrate the ability of intestinal microflora to moderate the neuropathological effects of m-DNB. After a single oral dose of 20 or 25 mg/kg m-DNB in PEG, GF male rats (n = 15) displayed a marked ataxic response, in contrast to conventional rats (n = 12) or to GF rats that had been reseeded with a “cocktail” of intestinal microflora. Conventional rats did show ataxia when dosed with 20 mg/kg m-DNB each day for five days. GF rats accumulated approximately 20 and 13 times more radio-labeled m-DNB into the liver and brain, respectively, compared to conventional rats. As described by the authors, light and electron microscopy revealed that histopathological lesions of the brain were limited to the brain stem and inferior colliculus, although animals displaying these features were not necessarily those that had displayed the pronounced clinical signs. The histopathological lesions were considered similar to those typically brought about by thiamine deficiency, a well-documented feature of GF rats. The authors speculated that an observed rise in lactate in the damaged regions of the brains of treated GF rats might reflect interference by m-DNB of oxidative metabolism and pyruvate utilization. Subsequent research by the same group indicated that m-DNB interfered with intracellular redox mechanisms resulting in impaired glucose utilization (Philbert et al., 1987b). The main body of the report contained a detailed histopathological analysis of neurological lesions induced by an experimental protocol similar to that employed in their previous report (Philbert et al., 1987a). Light and electron microscopy revealed the formation of “bilaterally symmetrical vacuolated lesions (that) involve cerebellar roof, vestibular and

superior olivary nuclei and the inferior colliculi.” The authors considered the primary cellular targets to be astrocytes, oligodendrocytes and vascular elements, with secondary neuronal involvement. However, the precise mechanism of action of the compound has remained obscure (Philbert et al., 2000).

2.3.1.2 Mammalian Oral Toxicity – Subacute

Subacute studies involve repeated dosing of animals and parameters are measured at the end of a 14-day study duration. Reddy et al. (1994a) reported a 14-day study on the toxicity of m-DNB in five F344 rats/sex/group that was conducted to establish dosing levels suitable for longer-term studies. The compound was added to the diet to concentrations of 0, 2.5, 10, 25, 75, and 150 mg/kg, yielding respective doses that were calculated by the authors to be 0, 0.21, 0.8, 1.98, 5.77 and 10.56 mg/kg-day in males and 0, 0.22, 0.87, 2.02, 6.28 and 11.82 mg/kg-day in females. Clinical observations were made twice daily, food and water consumption twice weekly, while body weights were recorded at the beginning, weekly during the in-life phase of the study, and at termination. A complete profile of hematological and clinical chemistry parameters was assessed in blood samples obtained at necropsy. All tissues and major organs were observed for gross morphological lesions, and the weights of certain organs were recorded. Tissues were sampled, fixed and processed for histopathological examination. Slides of sections cut from high-dose and control tissues were examined under a light microscope. In addition, sections of certain potential target organs such as spleen and kidney (male only) were similarly examined for all groups.

There were no significant decreases in body weight in any of the treatment groups compared to control, although there was a comparative reduction in food consumption in both sexes of animals receiving m-DNB at the highest dose. Some dose-related changes in organ weight/body weight ratio were seen, including comparative increases in liver, spleen and kidneys, and reductions in testis. The lowest effective dose for responses such as these was 1.98 mg/kg-day, a level associated with relative increases in kidney weights in male rats, although this particular response may have been incidental to treatment since it did not appear to be part of a dose-response relationship. Though there were no obvious treatment-related changes in clinical chemistry parameters, most hematological indices were reduced compared to controls after 14 days of treatment. For example, there was a statistically significant reduction in hematocrit and erythrocyte count in female groups at a dose level of 2.02 mg/kg-day that appeared to be part of a dose-related response. Methemoglobin was significantly increased in females at a dose of 1.98 mg/kg-day and higher, and in males as 0.87 mg/kg-day and higher. Gross pathological signs were seen in the testes of male rats dosed at 5.77 mg/kg-day and higher and in the spleen of females at 2.02 mg/kg-day and males at 1.98 mg/kg-day. There were treatment-related histopathological changes in the bone marrow, spleen and brain of high-dose rats. In males, histopathological changes were noted in the spleen and kidneys of rats ingesting 1.98 mg m-DNB/kg/day and above and in the testes of rats

ingesting 5.77 mg m-DNB/kg/day. These lesions were characterized in the kidneys by the occurrence of tubular degeneration and associated hyaline droplet formation and in the testis by seminiferous tubular degeneration, the appearance of cell debris, and the formation of multinucleate cells.

A number of possible NOAELs and LOAELs could be derived from this study. For example, a NOAEL of 0.8 and a LOAEL of 1.98 mg/kg-day would be appropriate, based on the histopathological changes in the kidney in male rats. However, the most sensitive endpoint in the study appeared to be the hematological changes and the formation of methemoglobin that were evident in male rats at a dose of 0.8 mg/kg-day which, yielded a NOAEL of 0.21 mg/kg-day (Table 2).

2.3.1.3 Mammalian Oral Toxicity – Subchronic

Reddy et al. (1995) conducted a 90-day toxicological study in which 15 F344 rats/sex/group received 0, 1, 6 or 30 mg m-DNB/kg in their diet, amounts calculated by the authors to be equivalent to doses of 0, 0.06, 0.35 and 1.73 mg/kg-day in males and 0, 0.07, 0.39 and 1.93 mg/kg-day in females. A full range of in-life, clinical chemistry/hematological (at 45 and 90 days), gross pathological and histopathological evaluations were carried out as described above for the 14-day study (Reddy et al., 1994a).

Critical findings included an increase in the average relative spleen weight and a reduction in relative testis weights, a reduction in the absolute testis weight and the onset of profound histopathological changes in the spleen, kidney and testes of high-dose groups. While sporadic changes in clinical chemistry parameters appeared not to be related to treatment, marked dose-dependent changes in hematological parameters were observed, including reductions in hemoglobin, hematocrit and erythrocyte counts in both sexes of high- and mid-dose rats, increases in platelet counts in high-dose females and in reticulocytes and methemoglobin formation in mid- and high-dose groups. These results indicated a NOAEL of 0.06 mg/kg-day for the hematological responses, with an associated LOAEL of 0.35 mg/kg-day.

The earlier report of Cody et al. (1981) described subchronic experiments in which m-DNB was administered to both sexes of Carworth Farms rats in drinking water. In one experiment, concentrations of 0, 50, 100 or 200 mg/L were provided to 6 animals/sex/group for 8 weeks, while in the second, 0, 3, 8 or 20 mg m-DNB/L were provided to 20/sex/group for 16 weeks. Average dose levels of 0, 4.72, 7.26 and 12.45 mg/kg-day in males and 0, 5.97, 9.0 and 24.43 mg/kg-day in females was calculated for rats in the 8-week study, based on the data in the report.

In general, dose levels of the 8 week study were associated with profound toxic responses, including four of six fatalities in high-dose males, two of six fatalities in high-dose females, reductions in body weight gain compared to controls that were probably over and above any anticipated reduction due to curtailed food and water intake. Reduced hemoglobin concentrations, enlarged spleen (except in high dose females) and atrophy of testes were in evidence at all m-DNB dose levels.

Average doses of 0, 0.4, 1.13 and 2.64 mg/kg-day in males and 0, 0.48, 1.32 and 3.1 mg/kg-day in females could be determined for rats in the 16-week study, based on the data in the report (Cody et al., 1981). Among the compound-related findings were reduced testicular weights with depleted spermatogenesis in high-dose males and enlarged spleens associated with a number of histopathological manifestations including hemosiderosis in all dose groups for males and in the mid- and high-dose groups for females. These findings suggested that a NOAEL of 0.48 mg/kg-day (with a related LOAEL of 1.32 mg/kg-day) would be appropriate for the spleen effects, which, appeared to be the most sensitive response to m-DNB. A NOAEL from this study (0.4 mg/kg-day) was used by the IRIS compilers to derive a reference dose (RfD) (human health) of 1×10^{-4} mg/kg-day for m-DNB (U.S. EPA, 2000).

Testicular impairment and indicators of reproductive success were the subject of a subchronic study on the toxicity of m-DNB in Sprague-Dawley rats (Linder et al., 1986; Perreault et al., 1989). Twelve male Sprague-Dawley rats/group were gavaged 5 days/week for 12 weeks with 0, 0.75, 1.5, 3.0 or 6.0 mg m-DNB/kg-day in a corn oil/acetone mixture. As a functional assessment of male reproduction, each male was mated with two virgin females after 10 weeks and pregnant females were then terminated on gestation day 21 and evaluated for reproductive parameters. All males were sacrificed after 12 weeks and a battery of sperm parameters were evaluated according to dose, including spermatid count, cauda reserves, percentage motility, morphology, histopathology and fertility. There was severe toxicity in those animals receiving 6 mg/kg-day m-DNB, as evidenced by ataxia, loss of balance, muscular rigidity, and a lower body weight. These effects became especially marked during the first week of breeding, to such an extent that breeding and m-DNB treatment were discontinued in this group after 4 days. However, there were no clinical signs of toxicity in subjects receiving 3 mg/kg-day m-DNB or less. In male rats sacrificed after 12 weeks, there were decreased testicular and epididymal weights at 3.0 mg/kg-day and increased spleen weights in animals receiving 1.5 and 3.0 mg/kg-day. The most sensitive parameter of testicular toxicity appeared to be the spermatid count, which showed a significant difference (72%) to controls at 1.5 mg/kg-day and above, indicating a NOAEL of 0.75 mg/kg-day and a LOAEL of 1.5 mg/kg-day. In addition to testicular toxicity, male reproductive success was deleteriously impacted by m-DNB. No litters were produced in females mated with males receiving 3.0 or 6.0 mg 2,3-DNB/kg-day, and significantly fewer pups compared to controls were produced as a result of matings of females with males receiving 0.75 and 1.5 mg/kg-day. This data indicates that m-DNB disrupts normal reproductive function in males and suggests a LOAEL of 0.75 mg/kg-day.

2.3.1.4 Mammalian Oral Toxicity – Chronic

No studies were identified that explored the toxicity of m-DNB through chronic exposure.

2.3.1.5 Mammalian Oral Toxicity – Other

A large number of experimental studies have demonstrated that m-DNB is a potent testicular toxicant. In fact, a single dose of m-DNB to male laboratory rodents has served as a model for the investigation of testicular impairment. For example, Linder et al. (1988) administered a single oral dose of 48 mg m-DNB/kg in acetone/corn oil (2% v/v) to eight male Sprague-Dawley rats/group and demonstrated a marked seminiferous tubular degeneration, with reduced testicular and epididymal weights, reduced numbers of sperm heads, degeneration of sperm tails, and the appearance of dead and “decapitated” cells. Subsets of treated animals were terminated after different time intervals up to 175 days, throughout which treated males were mated intermittently with untreated females. In line with the reduction in numbers and integrity of the sperm, some of the matings produced no fertilized eggs, for example, at 34–38 days post-treatment. However, some but not all treated males recovered their fertility in subsequent matings. A companion paper examined the detailed histopathological consequences of a single oral dose of 48 mg m-DNB/kg as used in Linder et al. (1988), showing the testis to be severely damaged at 24 hours post-treatment, with the appearance of “increased numbers of regressive seminiferous tubules that exhibited degenerating pachytene spermatocytes, chromatin margination in spermatids, deformed spermatid heads, retained spermatids and reduced numbers of meiotic figures” (Hess et al., 1988). The ability of spermatogenesis to recover from this chemical insult was variable, since only three of seven treated males were morphologically and functionally normal at 175 days post-treatment.

Blackburn et al. (1988) compared the ability of 1,2-, 1,3- and 1,4-DNB to induce testicular lesions in four male Wistar rats/group receiving a single dose of 50 mg/kg by gavage in PEG. Histopathological lesions of the testis were noted in those animals receiving m-DNB but not 1,2- or 1,4-DNB. This suggests a stereospecificity of the mechanism by which m-DNB induces testicular atrophy and sperm deficits, a feature not shared with the mechanism of methemoglobin induction in which 1,4-DNB was just as effective as m-DNB. In the dose response phase of their study, Blackburn et al. (1988) found single doses of 5 and 10 mg m-DNB/kg to be ineffective in inducing testicular lesions in Wistar rats, although testicular lesions limited to Stages VIII through XI of the spermatogenic cycle were evident 12 hours after a single oral dose of 25 mg m-DNB/kg and similarly, 48 hours after a single oral dose of 15 mg 1,3-DNB/kg.. The authors considered the ultrastructural evidence to implicate the Sertoli cells as the prime targets for the toxic action of m-DNB with germ cell damage a secondary event. This conclusion was endorsed by Rehnberg et al. (1988) who measured a range of testicular and serum hormone concentrations in male Sprague-Dawley rats (receiving a single gavage dose of 32 mg m-DNB/kg in corn oil) and found little if any evidence that the m-DNB-induced testicular lesions might be secondary to changes in testicular, brain or pituitary hormone levels.

A report by Evenson et al. (1989a) demonstrated that the testicular effects of m-DNB were also apparent in male adult but not prepubertal B6C3F1 mice exposed to the compound. They administered a

single gavage dose of 0, 8, 16, 32, 40 or 48 mg m-DNB/kg in corn oil/acetone and measured testicular responses at various time intervals for the next 25 days. The 48 mg/kg dose appeared to be the critical dose level for the onset of testicular effects in adult B6C3F1 mice, resulting in abnormal spermatogenesis, reduced testicular weights, altered germ-cell type ratios, abnormal chromatin structures and an increase in abnormal sperm head morphology. The same researchers exposed 16 male Sprague-Dawley rats/group to single doses of either 0, 8, 16, 32 or 48 mg m-DNB/kg and examined the compound's effects on spermatogenesis using flow cytometry, 1, 4, 16 or 32 days post-dosing (Evenson et al., 1989b). An increase in the incidence of unusual cell types was observed on day 1 after 48 mg/kg and on day 4 after 16 mg/kg or greater. The presence of haploid, diploid and tetraploid cells signaled a dose-dependent increase of germinal cell types as a consequence of treatment, elevated numbers of which persisted to post-treatment day 32 and beyond, often accompanied by aberrant sperm effects. Using Sprague-Dawley rats as well, Linder et al. (1990) observed a single dose no observed adverse effect level (NOAEL) of 8 mg/kg and a lowest observed adverse effect level (LOAEL) of 16 mg/kg for changes in epididymis weight, sperm head counts, caudal sperm reserves and sperm morphology.

Holloway et al. (1990) used *in vitro* fertilization to investigate the influence of m-DNB on Sertoli cells and thus the functional capacity of developing germ cells. Male Wistar (AP/ALPK) rats were given a single oral dose of either 0, 5, 15 or 25 mg m-DNB/kg in PEG, and sperm were collected from the cauda epididymis at various time points thereafter. The sperm was used in an *in vitro* fertilization technique through which reduced sperm fertilizing capacity was observed from 1.5 to 5 weeks and from 7.5 to 8.5 weeks after treatment with 15 and 25 mg m-DNB/kg and for 3, 5.5, 7.5 and 8.5 weeks after treatment with 5 mg m-DNB/kg. The authors interpreted their data to indicate that m-DNB did not affect all Sertoli cells equally but acted in a stage-specific manner, with Stages III, IX, XII and XIV appearing to be especially vulnerable to the toxicant. This stage-specific mechanism of m-DNB on the Sertoli cells was reported in a review by Nolte et al. (1995) and by McEuen et al. (1995) who obtained similar testicular damage in Sprague-Dawley rats irrespective of the route of administration of the compound (oral or intraperitoneal). Given the different concentrations of parent compound and metabolites that resulted from intraperitoneal versus oral dosing, McEuen et al (1995) speculated that only the duration of testicular exposure to the toxicant may govern susceptibility to toxicity once a threshold blood level of m-DNB is reached.

A number of studies have searched for compounds that might serve as plasma biomarkers of the incipient testicular damage induced by chemical toxicants such as m-DNB. Of a number of hormones and enzymes evaluated, the lactate dehydrogenase-C₄ (LDH-C₄) isoenzyme and the androgen binding protein (ABP) were shown to be viable candidates when single doses of 0, 10, 20 and 30 mg m-DNB/kg were administered to male Wistar (Alpk APfSD) rats and 25 mg m-DNB/kg was administered in a time course experimental protocol (Reader et al., 1991). Using the same experimental animal, Suter et al.

(1998) correlated histopathological lesions and ABP production to further implicate Sertoli cell dysfunction as the etiologically significant event in germ cell depletion.

Two research groups have employed morphometry to visualize the toxicological effects of m-DNB. Davis et al. (1994) administered a single oral dose of 0, 15 or 25 mg m-DNB/kg to male Sprague-Dawley rats, which then were sacrificed after 22 to 24 days. Collected sperm were measured under the light microscope for total width, interior width, and symmetry using automated sperm morphometry analysis, the studies revealing three subpopulations of m-DNB-affected sperm, two of which were abnormal to varying degrees. Ninety-three percent of sperm harvested from the untreated controls had a normal appearance, with dose-dependently lower proportions in m-DNB-treated groups (78% and 66% respectively, for the low- and high-dose groups, respectively). Matsui and Takahashi (1999) gavaged male Sprague-Dawley rats with a single dose of 0 or 25 mg m-DNB/kg, with groups of four rats being sacrificed 1, 2, 4 or 7 days after administration. Sertoli cells were examined under the microscope, and the toxicity of m-DNB was evaluated by counting the proportion of germ cells without abnormalities in each treatment group, according to four user-defined clusters of stages of the spermatogenic process. As described by the authors, a large number of vacuoles were seen in Sertoli cell cytoplasm one day after m-DNB administration. These changes were considered instrumental in the apoptotic cell death of both pachytene and diplotene spermatocytes, round spermatids and other germ cells associated with Sertoli cells.

2.3.1.6 Studies Relevant for Mammalian TRV Development for Ingestion Exposures

Acute, subacute, and subchronic experimental studies have delineated a distinct and well-defined range of toxicological effects of m-DNB. However, the range of animal models employed in these studies, predominantly F344 and Sprague-Dawley rats, may lend a degree of uncertainty to quantitative estimates of dose levels potentially protective of mammalian wildlife. In general, the use of a narrow range of experimental animals reduces confidence in whether the resulting NOAELs and LOAELs have necessarily captured the sub-threshold for mammalian wildlife.

While the IRIS compilers chose splenic enlargement and the formation of hemosiderin deposits as the principal effect of m-DNB for RfD derivation (U.S. EPA, 2000), a clear spectrum of other toxicological responses to m-DNB has emerged including (1) hematological effects including methemoglobinemia (2) nephropathy associated with cytoplasmic and/or hyaline droplet formation, (3) structural and functional impairment of the brain deficits, and (4) atrophy of the testis with associated degeneration of the seminiferous tubules and sperm.

Methemoglobinemia is reversible and can create a functional hypoxic blood condition. Carbon monoxide poisoning also creates a functionally similar condition. Humans with levels of COHb above 10% have reported symptoms of headache, yet the preponderance of adverse effects occur when COHb

concentrations exceed 2% (ACGIH 1997). Chronic congenital methemoglobinemia in humans has been found where 10-50% of circulating blood pigment is in the form of methemoglobin with subjects exhibiting no overt signs of toxicity (Smith 1996). Reddy et al. (1995) report differences between control and high dose rats of approximately 3% or less from m-DNB exposure. Given the uncertainty associated with the reported methemoglobin increase in these investigations, increased methemoglobinemia due to m-DNB was not considered biologically significant.

m-DNB also caused increased erythroid cell hyperplasia and pigmentation in the spleen. In addition, the data showed decreased hematocrit, red blood cell count, hemoglobin and an increase in mean cell volume in primarily the high dose groups, however, these values were within ranges considered normal for rats of that age group (Wolford et al., 1986). The combined effects of m-DNB on spleen histopathology and hematological parameters are indicative of anemia although the biological significance of these effects in these models are questionable.

There is uncertainty regarding whether the nephropathic changes and cytoplasmic droplet formation in the kidney in response to m-DNB represent the same phenomenon as the well described $\alpha_2\mu$ -globulin-mediated hyaline droplet formation that is typical of male F344 rats and those of other strains. For example, in the 90-day study on m-DNB, Reddy et al. (1995) found 9 out of 10 high-dose females but only 1 out of 10 high-dose males displaying cytoplasmic kidney droplets on histopathological examination, a finding that contrasts with the current understanding of the $\alpha_2\mu$ -globulin-mediated hyaline droplet phenomenon as a characteristic of male rats. For m-DNB, this weakens the analogy with the nephropathy displayed by 1,3,5-TNB in the subacute (Reddy et al., 1994b) and subchronic studies (Reddy et al., 1994c), which appears to have the “classical” $\alpha_2\mu$ -globulin-mediated hyaline droplet etiology. However, the cytoplasmic droplets identified in the kidney of F344 males in the 14-day study of m-DNB toxicity were clearly identified as hyaline (Reddy et al., 1994a). Contrasting these observations with those of 1,3,5-TNB-induced nephropathy in F344 rats in a 2-year study (Reddy et al., 1996) and those of m-DNB-induced nephropathy in the 90-day study (Reddy et al., 1995) (in each case with lesions that apparently did not resemble the typical $\alpha_2\mu$ -globulin-mediated hyaline droplet histopathology and gender-specific incidence) holds open the possibility that age-related morphological and chemical differences may exist between the kidney droplets formed in response to shorter periods of nitroaromatic dosing compared to those becoming manifest after a longer period of dosing. Although this response is common to nitroaromatic exposure, the impact of increased incidence of hyaline droplets in the kidney is unknown. Hence, given the uncertainty in etiology related to hyaline droplet formation and the lack of known biological/ecological significance, this endpoint cannot be used for derivation of the TRV.

Table 2. Summary of Relevant Mammalian Data for TRV Derivation

Study	Test Organism	Test Duration	Test Results		
			NOAEL (mg/kg/d)	LOAEL (mg/kg/d)	Effects Observed at the LOAEL
Linder et al. (1986) Perreault et al. (1989)	Rat (Sprague Dawley)	12-w	0.54	1.1	Reduced spermatid head count
			NA	0.54	Significant reduction in reproductive performance (pups/litter)
Philbert et al. (1987)	Rat (F344)	5-d	NA	20	Ataxia in 6/6 male rats.
Reddy et al. (1995)	Rat (F344)	90-d	0.07	0.35	Methemoglobinemia and an increase in reticulocytes
			0.39	1.73	Reduction in RBCs and in other hematological responses, changes in spleen and testicular histopathology
Reddy et al. (1994a)	Rat (F344)	14-d	0.21	0.8	Methemoglobin formation
			0.8	1.98	Enlargement of the spleen
			1.98	5.77	Nephropathy associated with hyaline droplet formation, testicular degeneration
Cody et al. (1981)	Rat (Carworth Farms)	8-w	NA	4.72	Enlarged spleen, fluctuation in hemoglobin levels, atrophy and histopathological lesions of the testes
Cody et al. (1981)	Rat (Carworth Farms)	16-w	0.48	1.32	Enlarged spleen
			1.13	2.64	Depleted spermatogenesis

NA = not applicable
RBC = red blood cell

m-DNB appears to be a neurological toxicant, with pronounced histopathological lesions induced in various regions of the brain as a consequence of acute dosing. While speculation on the mechanism of these effects has centered on (1) the possibility of impairment of oxidative metabolism and a perturbation of intracellular redox mechanisms and/or (2) changes in the concentrations and activities of various neurotransmitters and their metabolites, there is little solid evidence to indicate precisely how the observed effects are brought about (Philbert et al., 2000). The most pertinent study on the neurological effects of m-DNB was Philbert et al. (1987). However, in this study, rats showed signs of ataxia although only 6 conventional (non-germ free) rats were dosed with one concentration of m-DNB. The limited data and study design precludes an adequate assessment of the neurological effects of m-DNB. Although it is reasonable to infer that ataxia will likely result in decreased a lower survival, the lack of a dose-response regime and endpoints consistent with the other data suggest the questionable nature of these data for TRV derivation. Given these uncertainties associated with the whole-organism effects of the histopathological lesions and the limited availability of data, these studies alone cannot be used for derivation of the TRV.

Impairment of the male reproductive organs with associated decreases in sperm production and motility and reproductive performance is a consistent response of experimental animals to m-DNB (Linder et al., 1986; 1988; Blackburn et al., 1988; Evenson et al., 1989a; 1989b; Holloway et al., 1990). As described in Section 2.3.1.1, a single dose of m-DNB of 5 mg/kg-day or more is sufficient to induce testicular effects and sperm deficits in various strains of experimental animals (Holloway, et al., 1990). In the most pertinent study (Linder et al., 1986), the NOAEL was 0.75 mg/kg for rats exposed to m-DNB in the diet for 12 weeks. From an ecological perspective, the most significant effect was decreased reproduction in m-DNB dosed males. In fact, no litters were produced from non-dosed females mated with males that had been dosed with 3 and 6 mg/kg-day and the number of pups per litter was significantly less from females mated with males dosed with 0.75 and 1.5 mg/kg-day compared to females mated with control males. Although the effects of m-DNB on reproduction in males appears to be strong, limited data suggest that this response appears to be somewhat reversible on complete cessation of exposure (Linder et al., 1986). Nevertheless, given the severity of reproductive effects even from a single dose of m-DNB, decreased reproduction in males was chosen as the endpoint for derivation of the TRV.

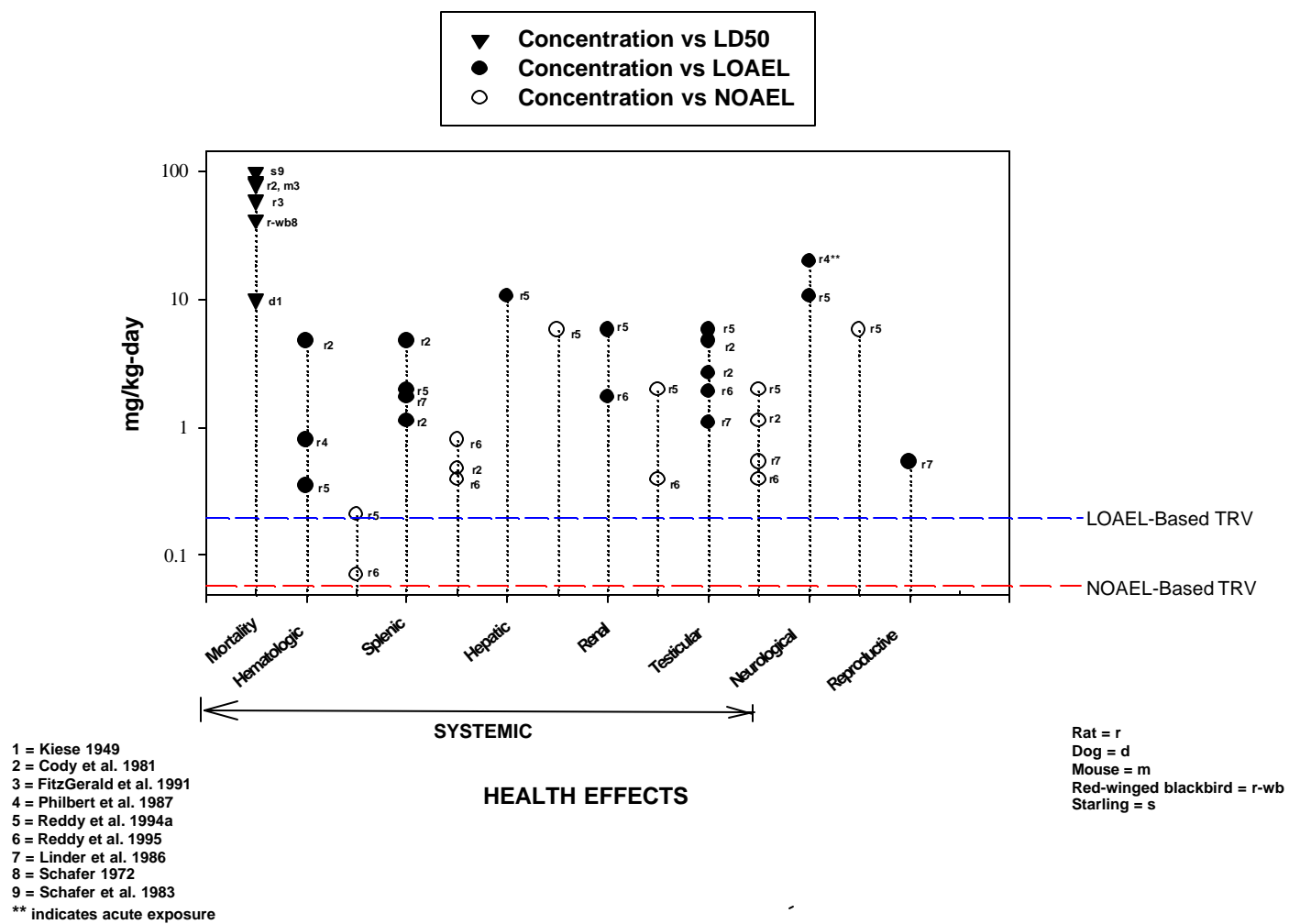
2.3.2 Mammalian Inhalation Toxicity

No inhalation studies conducted using mammals were found.

2.3.3 Mammalian Dermal Toxicity

No dermal studies conducted using mammals were found.

1,3-DNB HEALTH EFFECTS TO MAMMALS*



*Avian lethality data included

2.4 Summary of Avian Toxicology

2.4.1 Avian Toxicity - Oral

2.4.1.1 Avian Oral Toxicity - Acute

Researchers with the U.S. Fish and Wildlife Service (Schafer, 1972; Schafer et al., 1983) reported LD₅₀s for this compound in Red-winged Blackbirds (*Agelaius phoeniceus*) and European Starlings (*Sturnus vulgaris*) of 42 and >100 mg/kg, respectively. No other information was presented.

2.4.1.2 Avian Oral Toxicity - Subchronic

No data are available.

2.4.1.3 Avian Oral Toxicity - Chronic

No data are available.

2.4.1.4 Avian Oral Toxicity - Other

No data are available.

2.4.2 Avian Inhalation Toxicity

No data are available.

2.4.3 Avian Dermal Toxicity

No data are available.

2.5 Summary of Amphibian Toxicology

Toxicological data for the effects of m-DNB in amphibian species was not located. Ecotoxicological research on the effects of this compound in amphibians is recommended.

2.6 Summary of Reptilian Toxicology

Toxicological data for the effects of m-DNB in reptilian species was not located. Ecotoxicological research on the effects of this compound in reptiles is recommended.

3. RECOMMENDED TOXICITY REFERENCE VALUES

3.1 Toxicity Reference Values for Mammals

3.1.1 TRVs for Ingestion Exposures for the Class Mammalia

The parameters relevant to m-DNB toxicity involved changes in testicular histopathology and reproductive performance in male rats (Table 2.). The data relevant for TRV derivation for m-DNB is limited to subchronic oral exposure in rats. No chronic data are available and no repeated dosing studies have been conducted on mammals other than rats. Values used to derive the TRV were obtained from (Linder et al., 1986).

As outlined in Technical Guide 254, toxicity values used for derivation of a TRV should be based on potentially ecologically relevant effects. Effects of m-DNB on male reproduction was chosen as the parameter for TRV derivation based on decreased litter production from females mated with males exposed to m-DNB via oral gavage (LOAEL was 0.75 mg/kg-day, Linder et al., 1986). Also, this level at which reproductive effects occur is protective of the neurological effects noted by Philbert (1987).

Data on the toxicity of m-DNB is limited to two mammalian species, rats and mice, and no chronic toxicity studies were conducted. Although a study on m-DNB toxicity in shrews was available, it was of little use for TRV development because it provided information only on hemoglobin adduct formation, no controls were used and subjects were euthanized 24h after exposure. Given the above data limitations, the approximation approach was the only viable means for deriving the TRV for m-DNB (USACHPPM, 2000). An uncertainty factor of 20 was used to derive the NOAEL-based approximate TRV from a subchronic LOAEL of 0.75 mg/kg-day (Linder et al., 1986). An uncertainty factor of 4 was used to derive the LOAEL-based approximate TRV from the same subchronic LOAEL. Final values for the TRV were rounded. As stated, there are limited data for multiple species and no chronic toxicity data for DNB. However, the reported studies are of relatively high quality with a broad scope of observations that are consistent with each other and of other nitroaromatic compounds. It is for these reasons the TRV for m-DNB presented below was given a Medium confidence value.

Table 4. Selected Ingestion TRVs for the Class Mammalia

TRV	Dose	Confidence
NOAEL-based	0.04 mg/kg/d	Medium
LOAEL-based	0.2 mg/kg/d	Medium

3.1.2 TRVs for Inhalation Exposures for the Class Mammalia

Not Available at this time.

3.1.3 TRVs for Dermal Exposures for the Class Mammalia

Not available at this time.

3.2 Toxicity Reference Values for Birds

Only acute LD50 data are available for two species of birds, Red-winged Blackbirds and European Starlings. This information is originally presented in Schafer (1972) and no other supporting information was presented. Although the approximate method could be used to derive a TRV from these data, in the absence of details from the original reports, it is recommended that a TRV not be derived for birds until more information is available.

3.2 Toxicity Reference Values for Amphibians

Not Available at this time.

3.4 Toxicity Reference Values for Reptiles

Not Available at this time.

4. IMPORTANT RESEARCH NEEDS

The limited availability of data on the toxicity of m-DNB to wildlife species precludes the development of a high-confidence TRV. Hence, more studies on the toxicity of m-DNB to wildlife species are required. In particular, chronic toxicity studies on mammals and additional studies on non-mammalian wildlife such as birds, reptiles and amphibians are particularly warranted. More information regarding the toxicity of m-DNB to wildlife would likely allow the derivation of a high confidence TRV.

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APPENDIX A

LITERATURE REVIEW

The following files were searched in Dialog:

File 155 MEDLINE; File 156, TOXLINE, File 5 BIOSIS, File 10 AGRICOLA, File 203 AGRIS, File 399 Chemical Abstracts, File 337 CHEMTOX, File 77 Conference Papers Index, File 35 Dissertation Abstracts, File 40 ENVIRONMENTAL, File 68 Environmental Bibliography, File 76 Life Sciences Collection, File 41 Pollution Abstracts, File 336 RTECS, File 370 Science, File 143 Wilson Biological & Agricultural Index, File 185 Zoological Record, File 6 NTIS, File 50 CAB, File 144 PASCAL, File 34 SCISEARCH.

The search strategy for **Amphibians & Reptiles**:

- ◆ Chemical name, synonyms, CAS numbers
- ◆ AND (amphibi? or frog or frogs or salamander? or newt or newts or toad? or reptil? or crocodil? or alligator? or caiman? snake? or lizard? or turtle? or tortoise? or terrapin?)
- ◆ RD (reduce duplicates)

The search strategy for **Birds**:

- ◆ Chemical name, synonyms, CAS numbers
- ◆ And chicken? or duck or duckling? or ducks or mallard? or quail? or (japanese())quail?) or coturnix or (gallus())domesticus) or platyrhyn? or anas or aves or avian or bird? or (song())bird?) or bobwhite? or (water())bird) or (water())fowl)
- ◆ RD

The search strategy for **Laboratory Mammals**:

- ◆ Chemical name, synonyms, CAS numbers
- ◆ AND (rat or rats or mice or mouse or hamster? or (guinea())pig?) or rabbit? or monkey?)
- ◆ AND (reproduc? or diet or dietary or systemic or development? or histolog? or growth or neurological or behav? or mortal? or lethal? or surviv? or (drinking())water))
- ◆ NOT (human? or culture? or subcutaneous or vitro or gene or inject? or tumo? or inhalation or carcin? or cancer?)/ti,de
- ◆ NOT ((meeting())poster) or (meeting())abstract))
- ◆ NOT (patient? or cohort? or worker? or child? or infant? or women or men or occupational)
- ◆ RD

The search strategy for **Wild Mammals**:

- ◆ Chemical name, synonyms, CAS numbers
- ◆ And(didelphidae or opossum? or soricidae or shrew? Or talpidae or armadillo? or dasypodidae or ochotonidae or leporidae)or canidae or ursidae or procyonidae or mustelidae or felidae or cat or cats or dog or dogs or bear or bears or weasel? or skunk? or marten or martens or badger? or ferret? or mink? Or aplodontidae or beaver? or sciuridae or geomyidae or heteromyidae or castoridae or equidae or suidae or dicotylidae or cervidae or antilocapridae or bovidae arvicolinae or myocastoridae or dipodidae or erethizontidae or sigmodon? or (harvest()mice) or (harvest()mouse) or microtus or peromyscus or reithrodontomys or onychomys or vole or voles or lemming?
- ◆ AND (reproduc? or diet or dietary or systemic or development? or histolog? or growth or neurological or behav? or mortal? or lethal? or surviv? or (drinking()water))
- ◆ RD

All abstracts from the DIALOG search were reviewed and encoded in ProCite. When the search retrieved an appreciable number of hits, *keywords in context* were reviewed to minimize costs before any abstracts were downloaded (Tier 1). However, when only a limited number of studies were identified by the search, the abstracts were downloaded at the time of the search (Tier 2).

As noted in Section 2.1, 62 hits on m-DNB were obtained in the initial search, of which 45 were selected for abstract evaluation. Thirty of these articles and reviews were retrieved for this survey.